*ADMET & DMPK 3(3) (2015) 287-295; doi: **10.5599/admet.3.3.191***

ADMET

Open Access : ISSN : 1848-7718

http://www.pub.iapchem.org/ojs/index.php/admet/index

Original scientific paper

Prion protein and its interactions with metal ions (Cu^{2+}, Zn^{2+}, and Cd^{2+}) and metallothionein 3

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Received: June 10, 2015; Revised: August 02, 2015; Published: September 05, 2015

Abstract

The effects of heavy metals (Zn^{2+}, Cu^{2+}, and/or Cd^{2+}) on Escherichia coli expressing either prion ($hPrP^{C}$) or metallothionein 3 (MT-3) brain proteins capable of binding these metals were investigated. The expression of $hPrP^{C}$ or MT-3 in E.coli was confirmed using western-blot and dot-blot methods. After analyzing growth curves, we found that bacteria expressing prion protein better tolerated the presence of Zn^{2+} in comparison with wild-type bacteria and bacteria expressing MT-3. The addition of Cd^{2+} and Cu^{2+} was well tolerated by bacteria expressing MT-3, whereas the bacteria expressing prion protein displayed slower growth when compared to the wild-type. We subsequently determined total content of the MT in bacteria using differential pulsed voltammetry (DPV), and depending on the treatment of the individual metals. MT expression in MT3 transformed cells as well as in control E.coli cells increased at the lowest metal concentration (25 μM), followed by a decrease at higher metal concentrations (50, 75, and 150 μM). The highest increase by Cd^{2+} were observed. MT expression pattern in $hPrP^{C}$ transformed cells was different. After application of Cu^{2+} an increase in MT expression continued also at higher metal concentrations; and after application of Cd^{2+} and Zn^{2+} no decrease in MT expression at higher metal concentrations was observed.

Keywords

prion diseases; PrP^{C}; PrPSc; MT; MT-3; copper; zinc; cadmium.

Introduction

Numerous studies suggest that prion disease is caused by conformational conversion of PrP^{C} (the normal cellular prion protein) to PrP^{Sc} (its abnormal isoform), which becomes infectious [1]. In 1982, Stanley B. Prusiner formulated Prion theory, recognizing the substance of which was the definition of the prion protein infectious particle that lacks nucleic acid [2]. PrP is encoded by a single copy of chromosomal gene [3]. The gene encoding PrP (*PRNP*) is a copy of the gene that is located on the 20th chromosome in humans [4]. Prion protein (PrP^{C}) is a protein naturally present in all mammalian cells. Cellular prion protein (PrP^{C}, Fig. 1A) is a glycoprotein that is located primarily in the central nervous system (CNS), but also occurs

in smaller quantities in other tissues. The physiological function of this protein is not yet fully understood, but it is probably involved in cell differentiation and synaptic transmission [5]. Posttranslational modifications of PrP^{C} and conformational changes to pathological isoform PrP^{Sc} are the molecular basis of prion diseases [6]. In comparison with the knowledge about PrP^{C}, very little is known about PrP^{Sc}. The pathological isoform (PrP^{Sc}, Fig. 1B) is highly resistant to degradation processes in the cells and, upon binding to PrP^{C} proteins these isoforms will change the original conformation to the conformation of PrP^{Sc}.

The presence of hardly degradable protein and lack of physiological PrP^{C} therefore will lead to the emergence and development of progressive neurodegenerative diseases known as transmissible spongiform encephalopathy (TSE), which are clinically heterogeneous and currently incurable [7-9]. These illnesses - in humans and animals - include the following diseases: Creutzfeldt-Jakob disease (CJD) or Gertsman-Straussler-Schenker syndrome in humans, scrapie in both sheep and goats, and bovine spongiform encephalopathy (BSE) in cows. [10-12]. Experiments with tissue culture of cerebellum showed that cells lacking PrP^{C} are more susceptible to oxidative stress and readily undergo cell death [13]. Some of advances in prion protein detection can be found in [14-19].

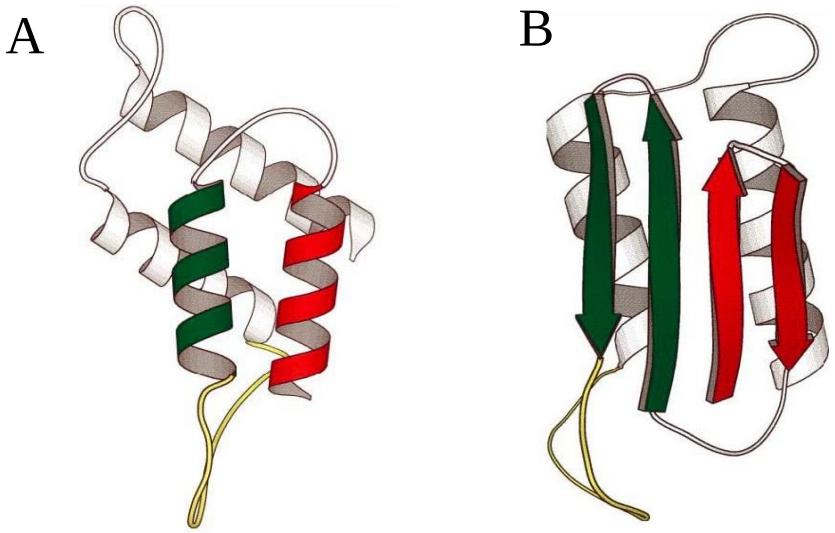

Figure 1. (**A**) Scheme of the structure of cellular form of prion protein (PrPC). (**B**) The structure of pathological (abnormal) prion protein (PrPSc). Adapted from [2].

Because the PrP^{C} is a metal binding protein, there is a hypothesis that the metal ions (Cu^{2+}, Zn^{2+} and Mn^{2+}) can play a role in the transformation of PrP^{C} to PrP^{Sc}. Equally important link between prion diseases and metals is a protein called metallothionein. The brain specific form of metallothionein-3 (MT-3) ensures the maintenance of optimal concentrations of metals in the brain. Also the involvement of MT-3 in the formation of neurodegenerative diseases was observed as the decrease in the MT-3 levels led to the formation of neurofibrillary clusters characteristic for neurodegenerative diseases [20-22].The aim of this study was to monitor heavy metal ions influence on the growth and electrochemical properties of *E. coli* strains transformed with PrP^{C} or MT-3 protein.

Experimental

Chemicals, preparation of deionized water and pH measurement

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) in American Chemical Society (ACS) purity unless noted otherwise. The deionized water was prepared using reverse

osmosis equipment Aqual 25 (Aqual s.r.o., Brno, Czech Republic). The deionized water was further purified at 18 MΩ resistance using MiliQ Direct QUV equipped with UV lamp. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

Chemical transformation, growth conditions and isolation of protein fraction

The Human MT-3 protein (hMT-3) and human prion protein ($hPrP^C$) were cloned in the pRSET-B vector (Invitrogen, USA). High levels of expression of DNA sequences cloned into the pRSET vectors were made possible by the presence of the T7 promoter. The chemical transformation protocol was performed following the instructions of New England Biolabs, using as host BL21 (DE3) pLysS chemically competent *E. coli* strain. Bacteria transformed with pRSET-hMT-3 or pRSET-$hPrP^c$ plasmid were selected by ampicillin resistance. The positive transformants were confirmed by PCR screening.

The positive transformants were grown in LB (Luria Bertani) medium (10 g tryptone, 5 g yeast extract, 5 g NaCl) with 50 μg mL^{-1} ampicillin and 35 μg mL^{-1} chloramphenicol shaking at 37 °C overnight. The next day, each culture was grown to 0.1 of OD_{600nm}. When the culture reached the exponential phase (0.4 - 0.6), IPTG (isopropylthio-b-D-galactopyranoside) was added to a final concentration of 1 mM and the cells were allowed to grow for 4 to 6 hours. After centrifugation at 4000 rpm for 10 min, the pellet was resuspended, with 20 mM phosphate buffer at neutral pH and frozen in liquid nitrogen. The frozen lysate was thawed at 42 °C (this freeze-thaw action was repeated three times). The protein fraction was harvested by centrifugation at 4000 rpm for 10 minutes at 4 °C.

Measurement of growth curves after addition of selected heavy metal ions (Cu^{2+}, Zn^{2+} and Cd^{2+}) to the bacterial cultures

Procedure for the evaluation of the antimicrobial effect of tested compounds and their combinations consisted of measuring the absorbance using the apparatus Multiscan EX (Thermo Fisher Scientific, Germany) and subsequent analysis in the form of growth curves. Control bacterial cultures of *E. coli* and *E. coli* with hMT-3 or $hPrP^C$ were cultivated in LB medium with 50 μg mL^{-1} ampicillin, 35 μg mL^{-1} chloramphenicol and 1 mM IPTG for 24 hours with shaking. In the microplate these cultures were mixed with cadmium, zinc and copper ions (10, 50, 150 and 300 μM concentrations) or strains without addition of heavy metal ions alone as a control for measurements. Total volume in the microplate wells was always 300 μL. Measurements were carried out at time 0, then each half-hourly for 24 hours at 37 °C and a wavelength of 620 nm.

Preparation of samples

Each sample (control strains – E. coli, E. coli – MT-3, E. coli - $hPrP^C$ or strains with addition of 10, 25, 50, 75, 150 and 300 μM concentrations of Cu^{2+}, Zn^{2+} and Cd^{2+} ions) was centrifuged at 8000 rpm for 10 minutes. Liquid nitrogen was added to the pellet. After evaporation, 1 mL of phosphate buffer (pH 7) was added and samples were mixed for 30 minutes. Two minutes of ultrasound were used for the lysis of cells. After centrifugation at 8000 rpm for 10 minutes, the supernatant was used in the following experiments. For electrochemical measurements 25, 50, 75, and 150 μM of metal ions were used.

Electrochemical measurement of metallothionein by differential pulse voltammetry

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 693 VA Processor and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes, a cooled sample holder, and measurement cell cooled to 4 °C (Julabo F25, Germany). A

hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and platinum electrode was auxiliary. For data processing VA Database 2.2 by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 %) saturated with water for 120 s. Brdička supporting electrolyte containing 1mM $Co(NH_3)_6Cl_3$ and 1M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of -0.700 V, end potential of -1.750 V, modulation time 0.057 s, time interval 0.2 s, step potential 0.002 V, modulation amplitude -0.250 V, Eads = 0 V, volume of injected sample: 20 µl, volume of measurement cell 2 ml (20 µl of sample and 1980 µl Brdicka solution) for calibration curves. For calibration curve measurements MT2A standard (Ikzus Proteomics, Italy) was used. The volume for the measurement of bacterial culture of *E. coli* and *E. coli* with MT-3 or $hPrP^C$ with metals was 100 µl of bacterial culture and 1900 µl of Brdicka solution.

Statistical analyses

Software STATISTICA (data analysis software system) version 10.0 (Tulsa, Oklahoma, USA) was used for data processing. Half-maximal concentrations (IC_{50}) were calculated from logarithmic regression of sigmoidal dose-response curve. General regression model was used to analyse differences between the combinations of compounds. To reveal the differences between cell lines, Tukey's *post hoc* test within homogenous groups and also F-test was employed. Unless noted otherwise, $p < 0.05$ was considered significant. Data from 3 measurements were analysed.

Results and Discussion

The aim of this study was to determine the effect of copper, zinc and cadmium ions on metallothionein level and on the growth of two different *E. coli* bacterial cultures transformed with the plasmid containing *$hPrP^C$* or *MT-3* gene, and control *E. coli* (BL21(DE3)) strain. Proteins PrP^C and MT-3 are able to bind heavy metals, thus protecting cells from their toxicity and we attempted to test this hypothesis by the mentioned experiments.

Verification of the expression of recombinant human prion protein ($hPrP^C$) and metallothionein 3 (MT-3) using western-blot

The presence of the *$hPrP^C$* expression was verified using western-blot (Fig. 2). To achieve better production and stimulation of proteins in bacterial cells IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to culture. Bacterial cells, which were expressing $hPrP^C$ protein, were divided into pellet and supernatant after lysis and subsequent centrifugation. After electrophoretic separation by SDS-PAGE (Fig. 2A), the proteins were transferred to a nylon membrane where they were detected with antibodies against $hPrP^C$ protein. Positive band of $hPrP^C$ protein with size of about 20 kDa was detected in the pellet of bacterial cells in a sample with the addition of IPTG only. This finding can be explained by possible aggregation and crystallization of $hPrP^C$ in pellet (Fig. 2B). These results were also confirmed by dot-blot (not shown).

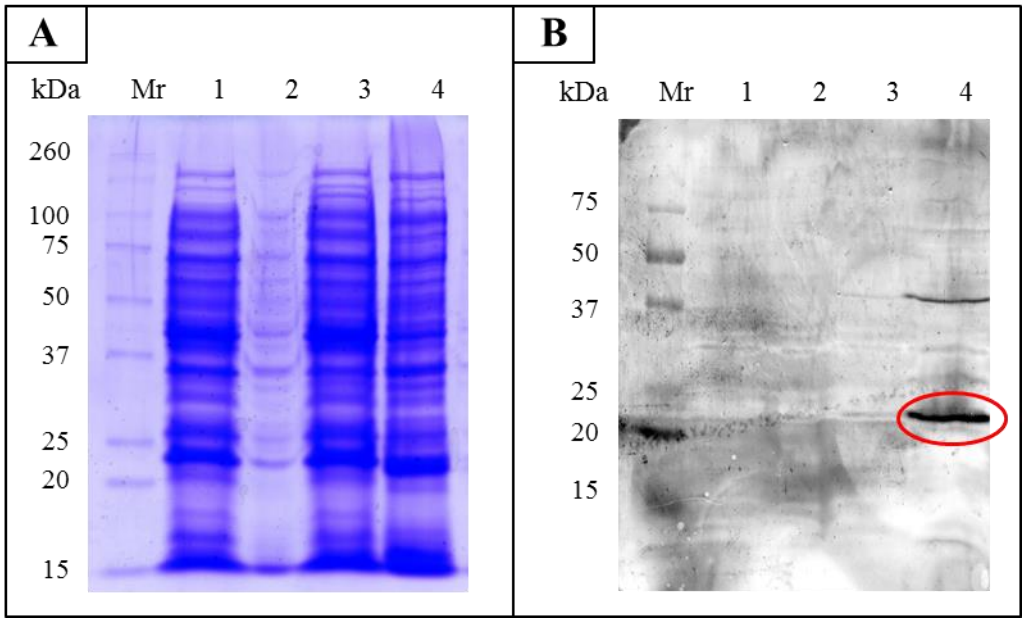

Figure 2. Verification of the prion protein (hPrPC) presence in *E. coli* BL21(DE3) cells transformed with a plasmid containing hPrPC gene. Red ellipse indicates occurrence of prion protein (size 20-23 kDa). (**A**) Gel stained with Coomassie brilliant blue, standard: Precision Plus Protein Dual Xtra Standards (Bio-Rad); (**B**) Western blot, standard: Precision Plus Protein Dual Xtra Standards (Bio-Rad) Mr-molecular mass standard. For both (A and B), lane 1: hPrPC supernatant, lane 2: hPrPC pellet, lane 3: hPrPC /+IPTG supernatant, lane 4: hPrPC /+IPTG pellet.

In the same way, the presence of MT-3 protein expression was verified (Fig. 3A). Using western-blot a positive band with a size of about 10 kDa in both cell fractions (supernatant and pellet) supplemented with IPTG was verified (Fig. 3B). The same results were achieved also by dot-blot method (not shown).

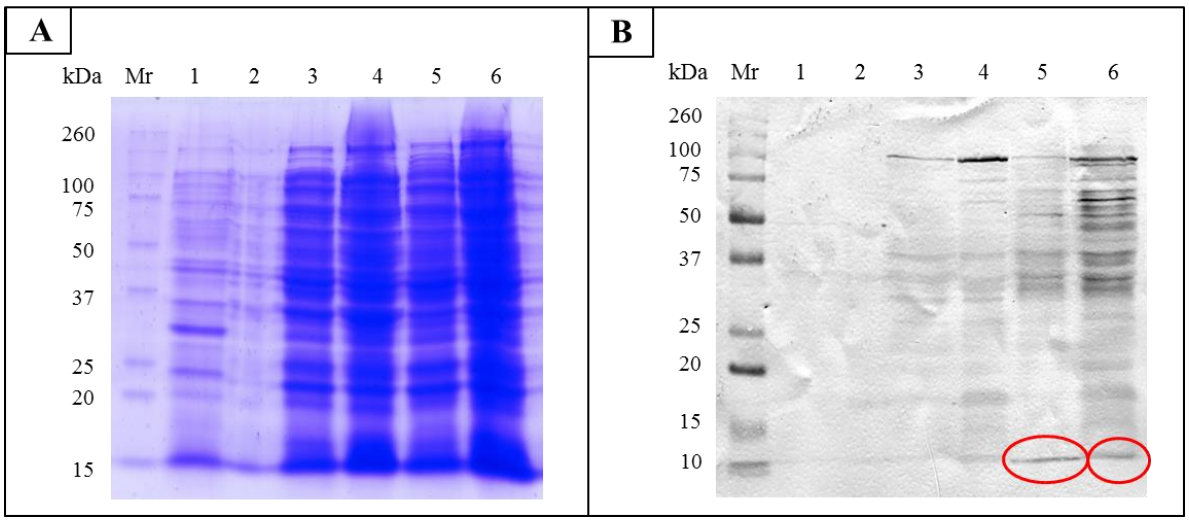

Figure 3. Verification of the metallothionein (MT-3) presence in *E. coli* BL21(DE3) cells transformed with a plasmid containing MT-3 gene. Red ellipses indicate occurrence of metallothionein 3 protein (size 7-10 kDa). **(A)** Gel stained with Coomassie brilliant blue, standard: Precision Plus Protein Dual Xtra Standards (Bio-Rad); **(B)** Western blot, standard: Precision Plus Protein Dual Xtra Standards (Bio-Rad), Mr-molecular mass standard. For both (**A** and **B**), lane 1 *E. coli* BL21(DE3) supernatant, lane 2: *E. coli* BL21(DE3) pellet, lane 3: MT-3 supernatant, lane 4: MT-3 pellet, lane 5: MT-3/+IPTG supernatant, lane 6: MT-3/+IPTG pellet.

The effect of metal ions (Cu^{2+}, Zn^{2+} and Cd^{2+}) on growth of bacterial cultures expressing hPrP^C or MT-3 proteins

An effect of copper, zinc and cadmium ions on growth of bacterial cultures expressing hPrP^C or MT-3 proteins was investigated by growth curve method. The procedure for evaluating antimicrobial activity of three chosen heavy metals (Cu^{2+}, Zn^{2+} and Cd^{2+}) was based on measuring the absorbance of transformed bacterial cultures of *E. coli* expressing the hPrP^C and MT-3 proteins compared to control bacterial *E. coli* BL21(DE3) culture, which was also exposed to the action of mentioned heavy metals. Figure 4 shows the effect of metal ions (Cu^{2+}, Zn^{2+} and Cd^{2+}) on the proliferation of transformed bacterial cultures expressing *hPrP^C* or *MT-3* genes.

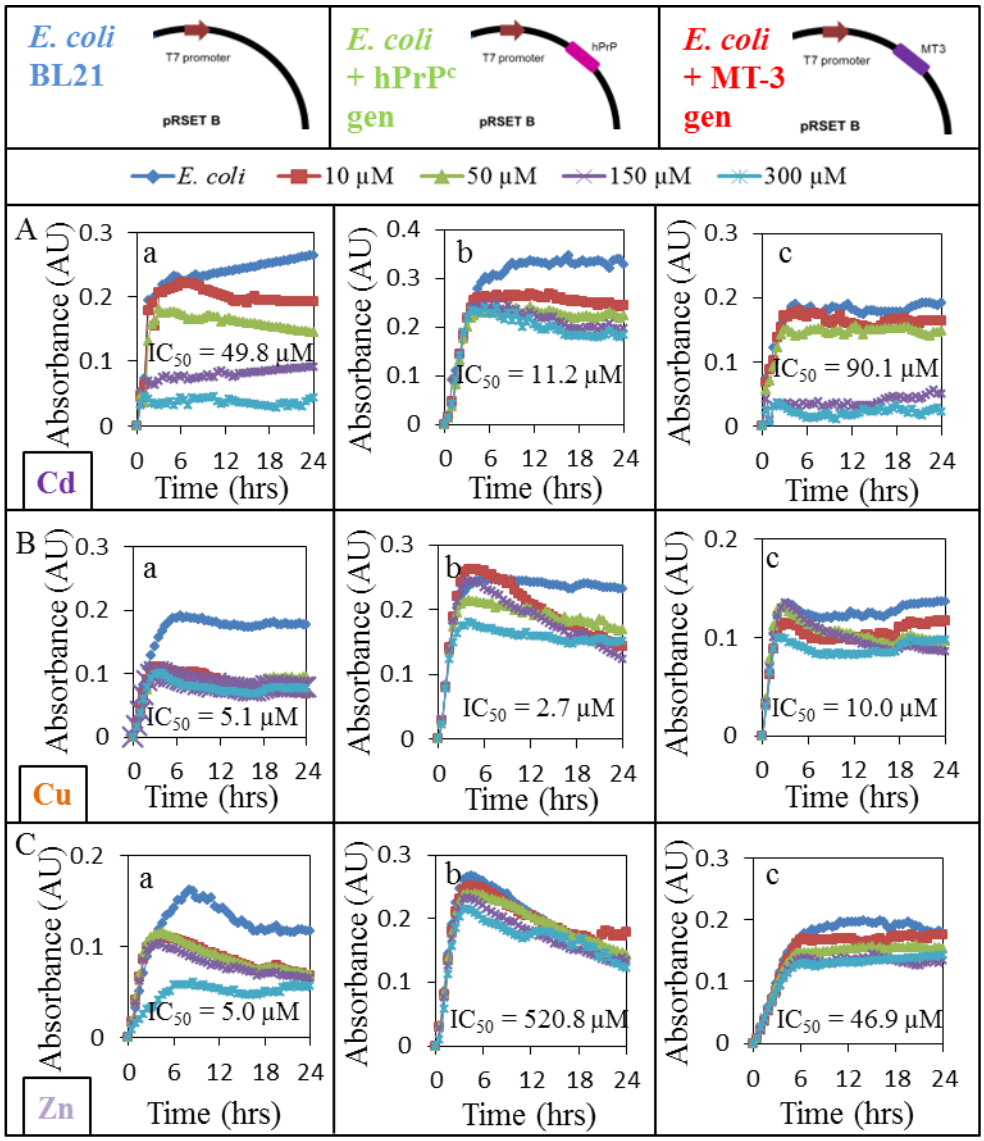

Figure 4. Spectrophotometric determination of growth curves obtained after treatment of *E. coli* BL21 cells transformed with an empty plasmid (**a**), plasmid containing hPrP^C gene (**b**) or MT-3 gene (**c**) with 0, 10, 50, 150 and 300 µM concentration of metal ions Cd (**A**), Cu (**B**) and Zn (**C**). Average values IC 50 (50 % inhibitory concentration) calculated from all growth curves are given. Data represent mean calculated from 3 measurements.

Cells, which expressed MT-3 protein and were exposed to the increasing concentration of cadmium ions, exhibited more effective protection against toxic effects induced by cadmium(II) (IC 50 = 90.1 µM) than cells expressing hPrP^C protein (IC 50 =11.2 µM) (Figs. 4A-a,b, c). On the other hand, the application of

copper(II) ions caused on the cells expressing $hPrP^C$ higher inhibition of growth (IC 50 = 2.7 µM) and growth was decreased by 46 % in presence of 300 µM concentration of copper (Figs. 4B-a;b). The concentration required for growth inhibition of 50 % of cells was measured on 5.1 µM and the highest used concentration of copper (II) caused 56 % growth inhibition of cells (Fig. 4B-a). The best protection against copper (II) toxicity showed cells expressing MT-3 (IC 50 = 10.0 µM) with 37 % inhibition of bacterial growth (Fig. 4B-c).

Similar findings as in the presence of cadmium (II) ions were also observed in the case of zinc (II) ions. The cells expressing $hPrP^C$ protein were exposed to the increasing concentration of zinc(II) ions. The best protection against toxicity of metals was observed after addition of zinc (II) ions (IC 50 = 520.8 µM), as it is shown in Figs. 4C-a,b. These bacterial cultures were inhibited by treatment with zinc (II) ions. The cells expressing MT-3 (IC 50 = 46.9 µM, Figs. 4C-c) were less protected (25 % inhibition of bacterial growth in presence of 300 µM concentration of zinc (II) and the least protected were the control cells (IC 50 = 5.0 µM, Fig. 4C-a). The control cells were inhibited by up of 69 % after addition of the same concentration of zinc (II) ions.

Electrochemical determination of the total MT level after addition of metal ions (Cu^{2+}, Zn^{2+} and Cd^{2+}) to the bacterial cultures expressing $hPrP^C$ or MT-3 proteins

Before the measurement of total MT level, a calibration curve (Fig. 5A) was determined by measuring different concentrations of MT 2A standard (0.39 – 25.0 µg/ml), whereas DP voltammograms are shown in Fig. 5B. Catalytic signal Cat2 measured at the potential of -1.56 V linearly corresponded to the concentration of metallothionein. MT values obtained by reading from the calibration curve were recalculated to mg of protein determined in the sample.

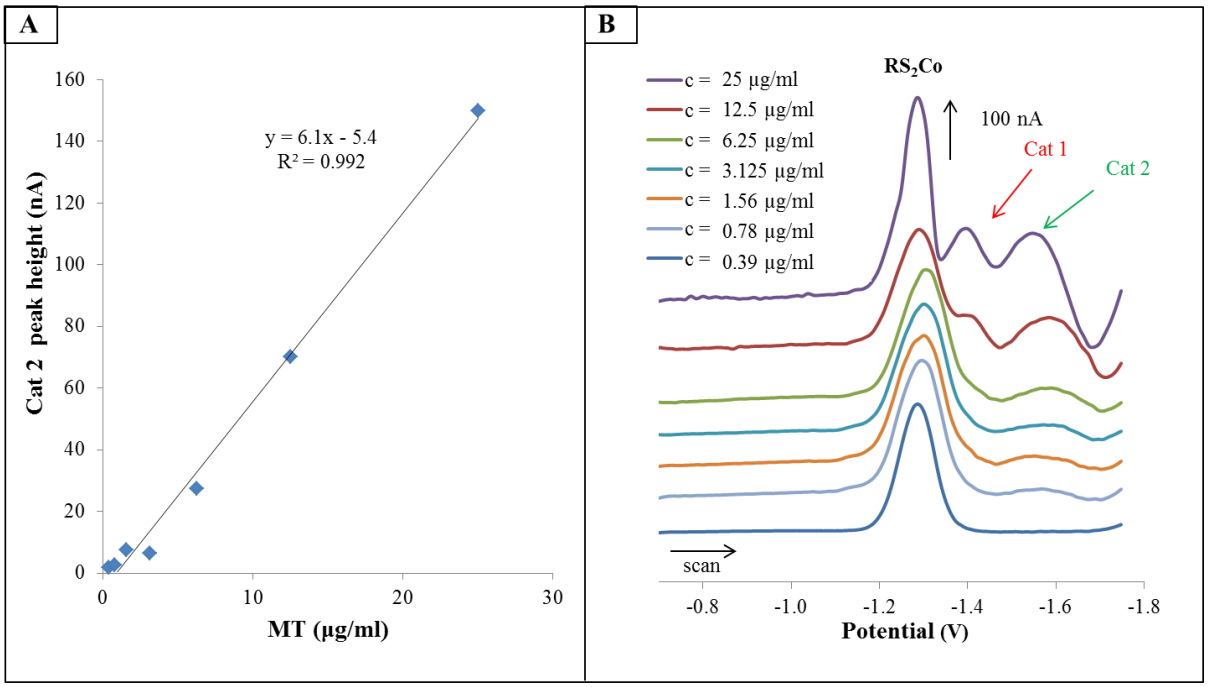

Figure 5. (**A**) Calibration curve of total MT determined using DPV method, as electrolyte Brdicka solution was used (1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonium buffer (NH_3(aq) and NH_4Cl, pH = 9.6)). Experimental parameters were chosen as follows: initial potential -0.4 V, end potential -1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, and modulation amplitude 250 mV. (**B**) DP voltammograms of MT standards (0.39 - 25 µg/ml) obtained from the calibration curve.

Further, DPV measurements of the total MT level in the control *E. coli* cells and in *E. coli* cells expressing $hPrP^C$ or MT-3 proteins were performed. These cultures were exposed to the increasing concentration of two essential metal ions (Cu^{2+} and Zn^{2+}) and one toxic metal ion (Cd^{2+}). Bacteria that expressed MT-3 protein, had slightly higher MT levels after application of increasing metal concentrations (25, 50, 75 and 150 µM) in comparison with control cells with similar pattern (Fig. 6). MT expression increased at lowest metal concentration (25 µM), followed by a decrease at higher metal concentrations (50, 75, and 150 µM). The highest increase in MT levels in bacterial cells after application of Cd^{2+} were observed followed by cells with addition of Zn^{2+}. The smallest increases in MT levels were observed in cells with addition of Cu^{2+}. MT expression pattern in $hPrP^C$ transformed cells was different. After application of Cu^{2+} an increase in MT expression continued also at higher metal concentrations; and after application of Cd^{2+} and Zn^{2+} no decrease in MT expression at higher metal concentrations was observed.

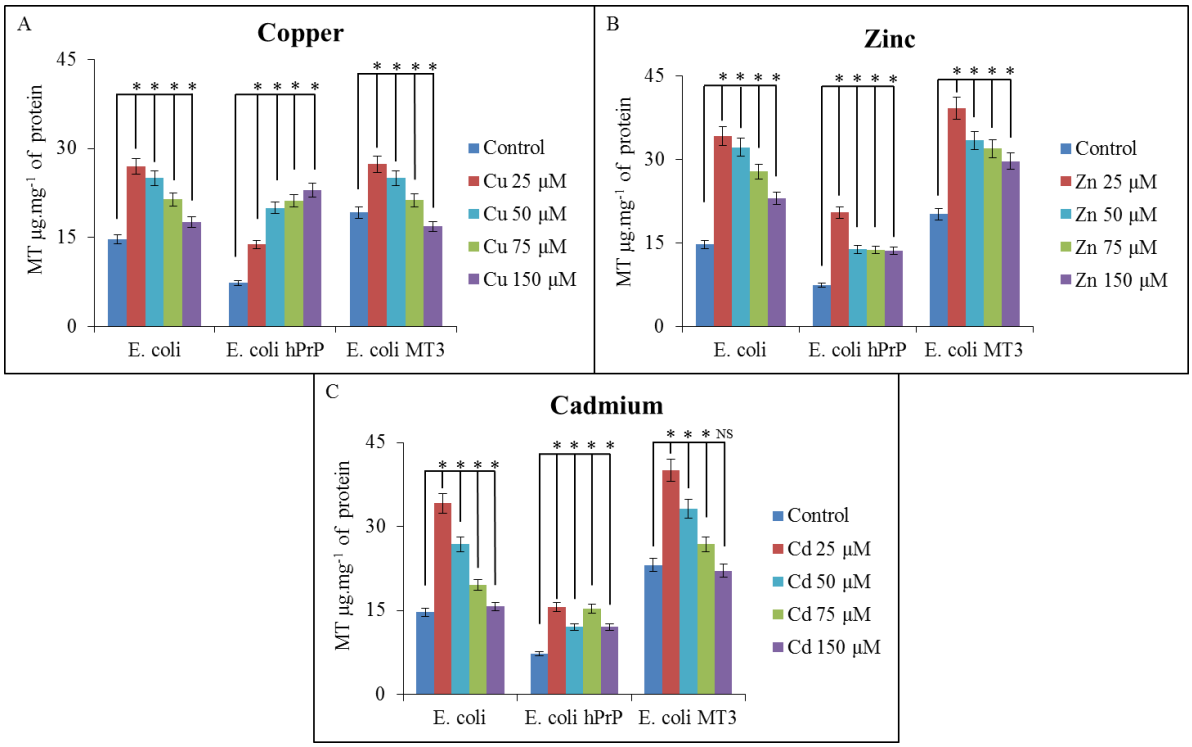

Figure 6. Metallothionein content in E. coli cells BL21 transformed with an empty plasmid, the plasmid containing $hPrP^C$ or MT-3 gene after incubation with different concentrations of (**A**) Cu, (**B**) Zn and (**C**) Cd in concentrations of 25, 50, 75, and 150 µM compared to control cells without addition of metal. Data represent mean ± s.d. calculated from 3 measurements, NS, not significant, *P < 0.05.

Conclusions

Clarifying the precise function of MT-3 and the conversion of the prion protein into its pathological form would contribute to a better understanding of the emergence of transmissible spongiform encephalopathies and the development of effective drugs to cure them. The first step for research of brain protein functions is the use of a bacterial model, where it is possible to observe the effect of metals on growth of bacterial cells, in which the brain proteins are produced. Another interesting topic is the monitoring of interconnection between functions of prion protein and MT-3.

In this work the presence of both $hPrP^C$ and MT-3 protein expression in bacterial cells was verified using western-blot and dot-blot. Furthermore, by the growth curve measurement and electrochemical

determination of MT protein levels, it was found that after addition of Zn^{2+} and Cd^{2+} MT-3 protein was more effective in protection of bacterial cells against toxicity of metals (Zn^{2+} and Cd^{2+}) than $hPrP^{C}$. The different situation occurred in the case of addition of Cu^{2+}, when the MT-3 was equally effective as $hPrP^{C}$, whose expression even increased the inhibitory effect of copper ions on the growth of bacterial cells.

***Acknowledgements**: Financial support by CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. The authors express their thanks to Vladimir Pekarik for the preparation of plasmids and to Yazan Haddad for a correction of the English language style.*

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